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**Preferred title:** Using fish models to investigate the links between microbiome and social behaviour: the next step for translational microbiome research?

**Running title:** Links between microbiomes and sociality in fish

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## Abstract

Recent research has revealed surprisingly important connections between animals' microbiome and social behaviour. Social interactions can affect the composition and function of the microbiome; conversely, the microbiome affects social communication by influencing the hosts' central nervous system and peripheral chemical communication. These discoveries set the stage for novel research focusing on the evolution and physiology of animal social behaviour in relation to microbial transmission strategies. Here, we discuss the emerging roles of teleost fish models and their potential for advancing research fields linked to sociality and microbial regulation. We argue that fish models, such as the zebrafish (*Danio rerio*), sticklebacks, poeciliids and cleaner-client dyads, will provide valuable insights into the roles of microbiome in shaping social behaviour and vice versa, while also being of direct relevance to the food and ornamental fish trades. The diversity of fish behaviour warrants more interdisciplinary research, including microbiome studies, which should have a strong ecological (field-derived) approach, together with laboratory-based cognitive and neurobiological experimentation. The implications of such integrated approaches may be of translational relevance, opening new avenues for future investigation using fish models.

Keywords: behaviour/sociality; cleaner fish; gut-brain axis; poeciliids; sticklebacks; zebrafish

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## **1. The gut-brain axis and its importance for health, stress physiology and behaviour**

The microbiota-gut-brain axis (MGB axis) has a pivotal role in host health, stress physiology and behaviour (Foster, Rinaman, & Cryan, 2017). The interaction between the gut microbiome and the brain is now well established, at least in mammals, and involves multiple pathways, including immune signalling or neurotransmission through bacteria-derived metabolites (such as serotonin, histamine and catecholamine regulators; Sandhu et al., 2017). In humans, neonatal microbial colonization of the gut is influenced by hospital environment, antibiotic use, birth delivery mode, breastfeeding vs bottle feeding, and early life stress (e.g. see Yang et al., 2016). Concomitantly with early establishment of the gut microbiome are key neurodevelopmental landmarks critical for establishing normal motor, cognitive and emotional function (Cong et al., 2016; Borre et al., 2014). The interaction between the microbiome, neurodevelopment and the central nervous system, i.e. the gut-brain axis, can be influenced by disease and stress, which cause an imbalance in the gut microbiome (DeGruttola, Low, Mizoguchi, & Mizoguchi, 2016; García-Ródenas et al., 2006). In healthy mammals, bacteria from the phyla Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria dominate the gut microbiome (e.g. Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012; Groussin et al., 2017; Yang et al., 2016). Disease-induced imbalances often result in increased intestinal permeability, due to the decrease in Firmicutes that are able to synthesize essential short-chain fatty acids (e.g. *Faecalibacterium* and *Eubacterium*), namely the butyrate, known to contribute to the maintenance of the gut barrier (Rivière, Selak, Lantin, Leroy & De Vuyst, 2016; DeGruttola et al., 2016). Likewise, imbalances induced by stress are often associated with increased corticotropin-releasing hormone, which also causes increased intestinal permeability (e.g. Vanuytsel et al., 2014). This means that in diseased and/or stressed hosts there is an increased ability of gut bacteria and their metabolites to translocate through the intestinal mucosa into the bloodstream, accessing immune cells and neurons in the enteric nervous system (Gareau, Silva & Perdue, 2008; Teitelbaum, Gareau, Jury, Yang & Perdue, 2008; Tognini, 2017). Indeed, several psychiatric disorders related to stress have been linked with increased intestinal permeability (e.g. chronic depression and anxiety, Kelly et al., 2015; Kelly et al., 2016; van de

Wouw et al., 2018) and in some cases diet supplementation with short-chain fatty acids was shown to ameliorate symptoms (e.g. van de Wouw et al., 2018). Depressive episodes, related to dysregulations of the hypothalamus-pituitary-adrenal (HPA) axis (Barden, 2004), also appear to have a close relationship with gut microbiome colonization. For instance, germ-free (GF) rats, that lack commensal gut bacteria – and, consequently, have a poorly developed immune system – exhibit elevated levels of plasma adrenocorticotrophic hormone and corticosterone (e.g. stronger HPA response) when subjected to restraint stress or to novel environments (Sudo et al., 2004; Clarke et al., 2013). In fact, neonatal stress leads to long-term changes in the diversity and composition of the gut microbiome, resulting in a reduction of beneficial bacteria (e.g. *Lactobacillus*, see Bailey and Cole, 1999), which could contribute to long-term changes in stress reactivity and in the stress-related behaviour observed in these rats. Treatment with probiotics (mainly *Lactobacillus* spp.) through the initial stress stage normalizes basal corticosterone levels, which are elevated after maternal separation (Gareau et al., 2008). Likewise, pre-treatment of rats with *Lactobacillus farciminis* reduces the intestinal permeability that results from restraint stress, as well as the associated hyperactivity of the HPA axis (Ait-Belgnaoui et al., 2012) and potential stimulation of the MGB axis. In addition to these findings relating microbiome and HPA axis function, there is direct evidence that the microbiome can influence central nervous system activity through the activation of stress-related neurons; for example, orally administered bacteria induce c-Fos expression in afferent vagal neurons (Lyte, Li, Opitz, Gaykema, & Goehler, 2006) and in paraventricular hypothalamic neurons in GF rats (Sudo et al., 2004) in the absence of systemic immune responses, suggesting that commensal and pathogenic bacteria in the gastrointestinal tract release signals to the central stress circuits. Moreover, electrophysiological experiments demonstrated that vagal nerve endings in the gut are less excitable in GF rodents (McVey Neufeld, Mao, Bienenstock, Foster, & Kunze, 2013), or more excitable after probiotic administration (Kunze et al., 2009), suggesting changes in enteric neuronal responses due to variations in commensal microbiome.

The reciprocal relationship between the microbiome and social life of hosts arose as a surprising topic, emphasizing the impact of microbial communities on animal social communication and signaling.

Microbial transfer between socially interacting partners is common and was recently reviewed by Archie and Tung (2015) and Vuong et al. (2017). Human families, for example, not only share a similar skin microbiome (e.g. Song et al., 2013; Lax et al., 2014), but pet dogs can facilitate homogenization of the microbiome between family members (Song et al., 2013). Besides the skin microbiome, oral and gut microbiomes were also more similar within a human family, and especially between sexually interacting partners (Song et al., 2013). Moreover, microbial transfer between interacting individuals can occur even when the time frame for direct contact is reduced, for example the skin microbiome of players from opposing teams after a roller derby tournament revealed a significant homogenization (Meadow, Bateman, Herkert, O'Connor, & Green, 2013). Analyses of the gut microbiome in non-human primates, the Verreaux's sifaka lemurs (*Propithecus verreauxi*), chimpanzees (*Pan troglodytes*) and baboons (*Papio cynocephalus*), showed that the best predictors for gut microbial composition were social group and physical interactions, in comparison to diet, kinship and habitat overlap (Moeller et al., 2016; Perofsky, Lewis, Abondano, Di Fiore, & Meyers, 2017; Tung et al., 2015). In birds, body contact also seems to be a good predictor for microbial diversity with higher diversities being positively associated with frequency of interactions in barn swallow (*Hirundo rustica*) females (Levin et al., 2016). In males this pattern was reversed, since frequent contact with other competing males caused significant stress, which, in turn, may have prompted a reduction in gut microbial diversity (Levin et al., 2016). Bacterial transmission can also be mediated through mating in kittiwakes (*Rissa tridactyla*), with White et al. (2010) demonstrating that the cloacal microbiome between mating partners is similar but can diverge over time if cloacas are covered thus preventing direct contact.

A general consensus is that commensal bacteria can affect social behaviour, potentially to maximize their transmission and colonization of new hosts (e.g. Archie & Tung, 2015), akin to host manipulation by macroparasites (e.g. Poulin, 2013). It is undisputable that bacteria can manipulate behaviour through the production of metabolites, which mediate chemical communication between interacting host partners (e.g. Ezenwa & Williams, 2014). Commensal bacteria are responsible for the production of odours that allow

sexual and reproductive state recognition (Theis et al., 2013) and can be determinants in mate choice (Sharon et al., 2010). Additionally, commensal bacteria may be key drivers in the cost-benefit calculus of group network interactions (e.g. Archie & Tung, 2015; Tung et al., 2015) and while in some cases social microbe transmission can benefit the host by promoting disease resistance (Endt et al., 2010; Koch & Schmid-Hempel, 2011; Stecher et al., 2010;), in others bacterial transfer might facilitate pathogen transmission (Elinav, Strowig, Henao-Mejia, & Flavell, 2011; Garrett, Gordon, & Glimcher, 2010).

Although research on microbial influence on animal social behaviour is vibrant and emergent, with much being discovered every year, most mechanistic pathways (neurophysiological for example) are unclear or unknown (see Vuong, Yano, Fung, & Hsiao, 2017). Furthermore, the focus of such studies has been mammalian models (reviewed by Pascoe, Hauffe, Marchesi, & Perkins, 2017). Here, we discuss the emerging roles of fish model candidates and their potential for advancing research fields linked to microbial regulation.

## **2. From human to fish: widening microbiome-based behavioural neuroscience**

### **2.1. Fish microbiome: what we know**

The most comprehensive review of teleost microbiome research collated 46 studies spanning 2006 to early 2014 that focused on the microbiomes of different mucosa in fish (see Table 1 of Llewellyn, Boutin, Hoseinifar, & Derome, 2014). These studies were mostly culture and/or 16S rRNA Sanger sequencing-based, focusing on hosts with high fishery, aquaculture or ornamental value. Most were purely descriptive or assessed the effects of diet (e.g. food substitution or supplements with prebiotics or probiotics) or host factors (ontogenetic, genetic or species-specific) on gut microbiota. Since 2014 the number of fish



microbiome studies has more than doubled (see for example Tarnecki et al. (2017) for a review on fish gut microbiome studies) and now the dominant phyla in fish microbiomes are well established and belong to Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes and *Fusobacterium* (e.g. Egerton, Culloty, Whooley, Stanton, & Ross, 2018; Wang, Ran, Ringø, & Zhou, 2017). While the focus of these new studies continues to be merely descriptive (e.g. Rosado, Pérez-Losada, Severino, Cable, & Xavier, 2019) or rather addressing the effects of diet supplements on gut microbiota (e.g. Ray et al., 2017; Wang et al., 2016), increased effort has been placed in finding which ecological factors are determinants of microbiome composition. For example, the effects of abiotic factors (e.g. salinity [e.g. Schmidt, Gomez-Chiarri, Roy, Smith & Amaral-Zettler, 2015], pH [e.g. Sylvain et al., 2016], season [e.g. Zarkasi et al., 2015] and geography [e.g. Wilkins, Rogivue, Schutz, Fumagalli, & Wedekind, 2015]) on fish microbiome composition are starting to be unraveled, including those driven by pollutants and habitat degradation (e.g. Narro et al., 2015).

Similar to mammals, the gut microbiome of fish seems to play an important role in the innate immune response (e.g. de Bruijn, Liu, Wiegertjes, & Raaijmakers, 2018; Gómez & Balcázar, 2008; Tarnecki, 2017;). Disentangling the links between microbiome and fish health is a hot topic, particularly given the growth of the aquaculture industry. In this respect, the effects of pathogens, antibiotics and probiotics on microbiome composition has been subject of several studies showing that significant changes take place (e.g. Carlson, Leonard, Hyde, Petrosino, & Primm, 2017; Gonçalves & Gallardo-Escarate, 2017; Hennersdorf et al., 2016; Llewellyn et al., 2017, Reid et al., 2017; Li et al., 2016).

The few studies that have focused on the effects of stress on fish microbiomes found a significant impact on skin microbial diversities. Aquaculture conditions, such as reduced oxygen, overcrowding and frequent fish handling (but see Box 2), can significantly reduce skin bacteria in farmed Atlantic salmon (*Salmo salar*) and Brook charr (*Salvelinus fontinalis*), namely the genera *Methylobacterium*, *Sphingomonas* and *Propionibacterium* that have antibiotic properties against fish pathogens (Boutin, Bernatchez, Audet, &

Derôme, 2013; Minniti et al., 2017,). Horizontal microbial transfer between fish in close physical contact has also been reported in the gut microbiome of fish, since differences associated with zebrafish (*Danio rerio*) genotypes were diluted when fish were co-housed (Burns et al., 2017).

## **2.2. A fishy absence: the gut-brain axis in fish**

As our understanding of fish host-microbiota interactions increases, so it becomes obvious that more research is necessary, particularly on the interdependence between the microbiota and fish behavioural responses. This contrasts with the rise in popularity of fish behavioural neuroscience, where several fish species are widely used as models to study social cognition and behaviour (Oliveira, 2013; Soares, Cardoso, Carvalho, & Maximino, 2018). While there is no doubt a significant bias towards using birds and mammals to study animal social behaviour and cognition, fish possess much more than just a predictable mixture of unlearned predispositions (Brown, 2015). Fish display sophisticated behaviours and complex social networks, such as Machiavellian strategies of deception and reconciliation (Bshary, Wickler, & Fricke, 2002), the ability to monitor the behaviour of others and their own reputation, to cooperate and to adjust their investment levels in accordance to context and even to use social tools to achieve direct goals (Brown & Laland, 2003; Soares, 2017; Soares et al., 2018). This complexity mirrors some homologous components of fish' neural representations with that of higher vertebrates (Oliveira, 2013). However, much remains to be discovered about the similarities and dissimilarities of behaviour and brain processes between fish and mammals.

## **3. The usefulness of fish models to study microbiome-behaviour interactions**

Given that fish display a complex array of social behaviours, from solitary individuals to complex group behaviour based on reciprocity, we should expect a variety of effects on (and of) the microbiome. An emergent example is the use of social network analysis to describe the structure of animal societies at

different organizational levels (i.e. individual, dyad, group, community, population, metapopulation and species). These methodologies, however, have their limitations (reviewed in Wilson, Croft & Krause, 2014). For example, direct observations of social interactions can be challenging in aquatic environments, and these studies often rely on tagged individuals, and having to control for tagging effects (Wilson et al., 2014). For this reason, the application of social network analysis has so far been restricted to: a) small and easy monitored freshwater teleost species (e.g. Croft et al., 2011; Croft, Krause & James, 2004;); b) to larger species that can be marked or recognized through individual-specific colour patterns and with behaviour that can be observed from a distance (e.g. elasmobranchs, Guttridge et al. 2011; Mourier, Vercelloni & Plane, 2012); or c) to those that may bear sophisticated acoustic or radio tags (e.g. Guttridge, Gruber, Krause & Sims, 2010). Currently, the challenge is to extend this methodology to other social fish systems, in the lab and in wild conditions, but also to include a stronger integrative approach, with, for instance, the addition of physiological and microbial measures, enabling the evaluation of social transmission combined with individuals' neurochemical balance and welfare (e.g. disease spread). Another key absence is the lack of knowledge regarding the gut-brain functioning in fish species and the potential impact on social behaviour. We argue that fish models, such as the zebrafish, sticklebacks, guppies, cleaner-client dyads (Box 1), can help provide valuable insights into the roles of microbiome in shaping social behaviour and vice versa.

### **3.1 Zebrafish**

The zebrafish is a key model organism in developmental biology and genetics and is increasingly used in behavioural neuroscience (Kalueff, Stewart, & Gerlai, 2014; Norton & Bally-Cuif, 2010; Stewart, Braubach, Spitsbergen, Gerlai & Kalueff, 2014). A wide array of tools, from behavioural assays to neurogenetic and neurogenomic tools (Gerlai, 2014; Rinkwitz, Mourrain, & Becker, 2011;) have been

developed for zebrafish, including specific assays for anxiety- and fear-like behaviour, as well as for stress responses (Clark, Boczek, & Ekker, 2011; Jesuthasan & Mathuru, 2008; Maximino et al., 2010; Stewart et al., 2014). So far, the single study focusing on the role of sociality in horizontal bacterial transfer in piscine hosts was conducted in zebrafish, with results supporting a role of co-housing in microbial composition of the gut (Burns et al., 2017). Furthermore, there is mounting evidence for a species-specific “core” gut microbiota in zebrafish, as animals reared for generations in the laboratory show similar components in relation to animals captured in the wild (Roeslers et al., 2011).

As described for other vertebrates, zebrafish exhibit ontogenetic shifts in microbiome composition (Falcinelli et al., 2015; Roeslers et al., 2011; Hall, Tolonen, & Xavier, 2017; Shin, Whon, & Bae, 2015). Recently, the effects of probiotics on zebrafish, namely on their behaviour, have been assessed. Adult zebrafish treated with the probiotic *Lactobacillus rhamnosus* IMC 501 for 28 days showed increases in the abundance of taxa belonging to Phylum Firmicutes in the gut (Borrelli et al., 2016). Treatment of zebrafish larvae with *L. rhamnosus* IMC 501 from 0 to 8 days post-fertilization decreased the relative abundance of Proteobacteria and increased Firmicutes. Borrelli et al. (2016) found similar increases in Firmicutes after 28 days of treatment in adult animals. Importantly, adult zebrafish treated with *L. rhamnosus* IMC 501 displayed less shoaling and increased brain-derived neurotrophic factor (bdnf), mRNA in the brain, with upregulation of genes involved in the serotonergic system in the brain (tph1a, tph1b, tph2, htr1aa, slc6a4a, and mao) and gut (tph1a) (Borrelli et al., 2016). The reductions in shoaling could represent an anxiolytic-like effect; however, Schneider et al. (2016) did not find alterations in anxiety-like behaviour after 15 days of treatment with *L. rhamnosus* GG – although this probiotic prevented the anxiolytic-like effect of chronic ethanol treatment. These discrepancies could also be due to differences in treatment duration (28 vs. 15 days). Consistent with this hypothesis, Davis et al. (2016) found that treatment with *Lactobacillus plantarum* for 30 days decreased anxiety-like behaviour in the novel tank test, an effect that was accompanied by increased slc6a4a (serotonin transporter) expression in the brain (but no alterations in the GABAergic, neuropeptide Y, or oxytocin-like pathways). *L. plantarum* did not alter gut microbiota diversity, but

Mycoplasmataceae, *Stenotrophomonas*, *Catenibacterium*, unclassified Lactobacillaceae, and *Achromobacter* were enriched in *Lactobacillus*-treated animals. Interestingly, when *L. plantarum*-treated animals were exposed to a chronic unpredictable stress paradigm, serum cortisol and leukogram alterations were not altered, but the probiotic protected against the gut dysbiosis induced by stress. Interpreting these findings is hard, as the authors did not report any behavioural alterations in stressed animals. For now results based on probiotic treatment should be treated with caution, but suggest that, at least in zebrafish, the microbiome is associated with defensive (anxiety-like) behaviour, and therefore could indirectly mediate sociality by altering the anti-predatory component of social behaviour or by decreasing the neophobia associated with social novelty.

### **3.2. Poeciliids**

The guppy (*Poecilia reticulata*) and indeed a range of other poeciliid fish are widely studied tropical fish species (Evans, Pilastro, & Schlupp, 2011), renowned models of ecology and evolution. The Trinidadian guppy system in particular has been used to study the complex role of gene flow on adaptation and fitness in a natural setting. Naturally isolated populations provide natural (replicated) study systems that have been used intensively to assess the impact of different environmental factors, and predation and parasitism pressure on host morphology, genotype and behaviour (e.g. Endler, 1995; Magurran, 2005; Reznick, Shaw, Rodd, & Shaw, 1977; Reznick & Ricklefs, 2009). While these wild guppies have been used to study micro-evolutionary speciation in field and laboratory studies, the ease with which they can be maintained and bred in captivity has meant they are also one of the most common fish in the world-wide ornamental trade. Their popularity relates to the availability of many different domestic colour and ornament varieties and hybrids (locally known as rainbowfish or millionsfish to reflect their colour and abundance). As the trade continues to expand, new strains are being selectively bred as these small viviparous fish have a fast generation time

and are relatively tolerant of poor water quality. This has provided the basis for numerous studies on the genetics, immunology, particularly the MHC-genes (e.g. Phillips et al., 2018) as well as the ecology and behaviour (Croft et al., 2011) of this model species.

Using the ‘natural Trinidad laboratory’ of replicated distinct up- and down-stream populations across four rivers, Sullam et al. (2015) were the first to assess the guppy microbiome, and they did so in relation to host adaptation. They found clear microbiome differences in the guts of wild-caught and laboratory bred fish, and from field specimens revealed that gut microbiome communities varied temporally, across streams and between ecotypes in a stream-specific manner. But this study also highlighted the myriad of other biotic and abiotic factors that could be driving microbiome divergence (Sullam et al., 2015).

Studies assessing the social behaviour of guppies have revealed how different life-history trajectories may shape sociality (e.g., Dugatkin, 1988; Farr, 1975) and the importance of cooperation in the establishment of these social groups (Croft et al., 2004; Dugatkin & Alfieri, 1991; Wilson et al, 2014). Social network analyses have shown that guppies occupy consistent social positions within a network (Krause et al., 2016) but that parasites can influence the guppy social networks (Croft et al., 2011). If the gut microbiome also influence’s guppy behaviour and there is a higher probability of microbial transfer between conspecifics, we predict, based on similar studies of zebrafish, that social interactions and prosocial behaviour will be associated with microbiome composition.

### 3.3 Sticklebacks

The three-spined stickleback (*Gasterosteus aculeatus*) is the temperate equivalent of the guppy, being a small freshwater fish that is robust and easy to maintain and breed in large numbers in both the laboratory

and field. But this fish is not just a leading model in ecology and evolutionary research; following publication of its genome (Jones et al., 2012) it arguably has reached supermodel status. Following glacial retreat in the north-ern hemisphere 12,000 years ago, repeated evolution from ancestral oceanic to derived freshwater forms resulted in many beneficial genetic variants surviving in thousands of distinct natural populations (Bell & Foster, 1994). Unlike laboratory genetic screening, which identifies mutants causing abnormal development, physiology or behaviour, these natural variants highlight biological innovations. Consequently, a large community of researchers utilises stickleback variants to understand adaptive speciation, behaviour, development, immunology (e.g. Cresko, McGuigan, Phillips, & Postlethwait, 2007; Huntingford, Ostlund-Nilsson, & Mayer, 2006; Scharsack, Kalbe, Harrod, & Rauch, 2007), and a range of projects are in progress assessing the microbiome of sticklebacks across a range of different environments. To date, such research has been largely confined to correlative approaches: QTL mapping, divergence mapping, and association studies.

Stickleback microbiome research has only just got off the starting block, but we already know that diet has a sex-specific effect on the gut microbiota (Bolnick et al., 2014b), and perhaps surprisingly individuals with more generalised diets have less di-verse microbiota than dietary specialists (Bolnick et al., 2014c). Of key importance is host genotype compared to transient environmental factors (Smith, Snowberg, Caporaso, Knight, & Bolnick, 2015), but systems-based approaches in the future are likely to reveal the true complexity of these interactions. Oceanic (ancestral) sticklebacks mount a strong immune response to residential microbiota compared to freshwater variants as revealed by the use of the stickleback as a newly developed gnotobiotic vertebrate model (Milligan-Myhre et al., 2016). Alongside zebrafish, sticklebacks could provide a powerful tool for mapping the natural genetic basis of the variation in immune response to microbes (Small, Milligan-Myhre, Bassham, Guillemin, & Cresko, 2017), and thus provide important clues regarding the role of intrinsic factors in microbiome composition. In the next few years, improved genome annotation will facilitate identification of candidate genes and experimental gene manipulation will provide the tools to validate suspected candidate genes. Importantly, recent research has already shown that specific

Major Histocompatibility Complex alleles and diversity are related to the composition and diversity of gut bacteria in sticklebacks (Bolnick et al., 2014a), as has previously been discerned for macroparasites (Matthews, Harmon, M'Gonigle, Marchinko, & Schaschl, 2010; Eizaguirre, Lenz, Kalbe, & Milinski, 2012).

### 3.4. Cleaner fish

Cleaning mutualisms are interspecific associations where cleaners remove ectoparasites and infected tissue from the body surface, mouth and gill chambers of their client fish (Côte, 2000). These interactions have been extensively studied, particularly in tropical regions, where obligate cleaner fish (cleaners that depend exclusively on client-gleaned material throughout their life cycle) occupy territories (known as cleaning stations) that are visited by fish species (often referred to as clients). Cleaner-client fish dyads are perfect candidate models to study the role of microbiome in mutualistic behaviour and physiology as both partners come in close physical contact during interactions, allowing direct microbe transmission (skin-to-skin) while potentially also modulating the gut microbiome of cleaners, which feed actively of clients' mucus, parasites and hyperparasites.

In recent years, a priority has been identification of neurohormonal candidates that may modulate behavioural changes in marine cleaning mutualisms, allowing us to relate social cognition to the underlying mechanistic and neural mechanisms (Soares, 2017). These studies have highlighted the importance of neuromodulators such as nonapeptide arginine-vasotocin (AVT), which has substantial motivational and learning effects on cleaners (Soares, Bshary, Mendonça, Grutter, & Oliveira, 2012a; Cardoso et al., 2015a, Cardoso, Paitio, Oliveira, Bshary, & Soares, 2012b). Monoamines such as serotonin and dopamine (DA) also have a crucial impact, with serotonin increases being responsible for changes in motivation to engage



mutualistically (Paula, Messias, Grutter, Bshary, & Soares, 2015), while disruption in dopamine transmission leads the cleaner to anticipate a lower probability of feeding or, alternatively, a higher likelihood of being punished by being chased or the client leaving (Messias, Paula, Grutter, Bshary, & Soares, 2016a). The effects of pharmacological stimulation of the DAergic system were most evident during the learning process, in which individual learning abilities of cleaners were tested in laboratory conditions (Messias, Santos, Pinto, & Soares, 2016b). The involvement of the dopaminergic system on cleaners' decision-making changed depending on context and the predictive condition of reward (for instance when time delays occur, Soares, Cardoso, Malato, & Messias, 2017a), or with familiarity/novelty (Soares, Santos, & Messias, 2017b). Moreover, the role of social stress on cleaning performance has been studied. Indeed, one of the most notable behaviours by these animals is the cleaning and entering of predators' mouths, a behaviour that has been interpreted as altruistic on behalf of the clients that may simply eat the cleaner (Trivers, 1971). This seemingly fearless behaviour became a paradoxical feature of cleaners' proactivity and 'gutsy' cooperative behaviour, but also of putative anxiety and stress control, particularly when cleaners were found to show a preference for predatory over herbivorous clients, despite no obvious increase in ectoparasitic crustaceans in these hosts (Soares, Cardoso, & Côté, 2007). The main hypothesis raised to explain this behaviour is that cleaners need to be more proactive and spend more time inspecting predators (therefore glean more parasites and other material), in response to an increase in cortisol levels of energetic expenditure and putative decrease of immune reactivity (Soares, Bshary, Cardoso, Côté, Oliveira, 2012b). The role of stress mechanisms mediating the cleaners' cooperative levels were further tested, as these mechanisms were discovered to be involved in cleaners' decisions, as the exogenous effects of cortisol rises were found to propitiate cleaners' behavioural switch from cooperation to cheating (e.g. more stress led to more cheating; Soares, Cardoso, Grutter, Oliveira, & Bshary, 2014).

Our current understanding of cleaner fishes' social behaviour and proximate mechanisms prompts us to hypothesise that the cleaners and clients' microbial composition may have a direct role in determining the costs and benefits of these complex behavioural trade-offs. But at this point it is impossible to know which

behaviours are influenced by cleaners' microbiota, especially given the diversity of social challenges cleaner must face.

#### **4. Concluding remarks**

The use of fish in microbiome-related research is expanding (Llewellyn et al., 2014), although currently the research is mostly ecological or diet focused on farmed or commercially valuable fish species (Box 2). Similar to mammals, the gut microbiome of fish seems to play an important role in the innate immune response (e.g. Gómez and Balcázar, 2008; Tarnecki et al., 2017; deBruijn et al., 2018), fish have also been shown to exhibit ontogenetic shifts in microbiome composition (Falcinelli et al., 2015; Roeselers et al., 2011; Hall et al., 2017; Shin et al., 2015), and there is also a diluting effect in microbiome differences caused by social proximity (Burns et al., 2017).

The use of fish as a complementary species in translational studies has been proposed before (Gerlai, 2014; Soares et al., 2018; Stewart et al., 2015) and one rationale for extending the breadth of model organisms in translational studies is that it increases the probability of finding shared mechanisms that represent “core” components of a given disease or function (van de Mooij-Malsen, Vinkers, Peterse, Olivier, & Kas, 2011; Gerlai, 2014). Recognized advantages of fish use in basic and applied neurobehavioural research include rapid generation time and fertility (typically external fertilization), small size, amenability to genetic manipulation, and finally intermediate physiological and behavioural complexity in relation to other model organisms (Friedrich, Genoud, Wanner, Ahrens, & Hughes 2013; Friedrich, Jacobson, & Zhu, 2010, Kalueff et al., 2012, 2014; Maximino et al., 2015; Norton & Bally-Cuif, 2010; Rinkwitz et al., 2011; Stewart et al., 2015; Soares et al., 2018). Moreover, complex neuroscientific tools and sophisticated behavioural paradigms have already been developed for the zebrafish model and behavioural studies of fish also cover a multitude of species. The combination of the laboratory tools available for zebrafish and the ecological and ethological perspectives gleaned from other fish species is therefore ideal (Hall, Serrano, Rodd, &

Tropepe, 2014). Importantly, protein-coding gene orthology between the zebrafish and human genome is close to 70% (Howe et al., 2013) and several genomic, transcriptomic, and transgenics tools have been developed for some fish (Rinkwitz et al., 2011).

A key difference between fish and other terrestrial models for microbiome studies is the fact that living in aquatic conditions may increase the level of microbiota contamination from surrounding water. However, many studies have compared the surrounding water microbiome with that of the fish, and while for larvae colonization from water microbiota seems to occur, for adult fish the observed differences are enough to discard this hypothesis (Califano et al., 2017; Larsen, Bullard, Womble & Arias, 2015; Schmidt, Smith, Melvin & Amaral-Zettler, 2015; Smith, Snowberg, Caporaso, Knight & Bolnick, 2015; Sylvain & Derome, 2017). At this point, the major drawback of our suggested systems is an overall lack of core information. So far microbiome research in fish disregards behavioural and physiological responses. Nevertheless, the potential of using fish models is endless considering their diversity, and the fact we may build from extensive knowledge regarding behavioural ecology (guppies, sticklebacks), physiology and genetics (cleaners and zebrafish) and animal production of certain key species. This opens the door for a wealth of research: for example, do most fish species share common behavioural and physiological end-points arising from modulation by microbiota?

Summarizing, the diverse behavioural systems exhibit by fish sets the stage for future interdisciplinary research that should start with a strong ecological (field-derived) approach, being followed by laboratory-based cognitive and pharmacological experimentation. The implications of such integrated approaches may be of translational relevance to human behavioural deficits and psychiatric disorders, especially regarding the influence of social conditions to microbiome functionality (see Box 2).

## Box list

Box 1. A move to therapy? The use of model fish species to unravel the effects of microbiome into human social behavioural disorders

Animal-focused research is pivotal to the advancement of scientific knowledge, but fish use in translational medical research has mostly focused on zebrafish. In neurosciences and behaviour, both larval and adult zebrafish have contributed to our understanding of the genetic control of brain processes, and the effects of pharmacological manipulations. In fact, the similarities between mammalian and zebrafish paradigms are a clear demonstration of the evolutionarily conserved nature of behaviour and physiological modulation across species (Kalueff et al., 2014). We expect fish to share an increasing role when it comes to microbiome-based research and, most specially, in contributing empirically to the influence of skin and gut microbiome on the onset and development of several human behavioural disorders.

The study of the microbiome of model fish organisms could generate interesting insights into conserved and derived features of host-microbiome interactions and their impact on behaviour. Importantly, while the study of fish models is likely to be useful in the study of the evolutionary biology of host-microbiome interactions, the availability of behavioural assays for anxiety in both adult and larval zebrafish offers potential biomedical applications. The development of models for stress-induced dysbiosis, novel developments regarding the zebrafish inflammasome (Angosto & Mulero, 2014), and the interactions between these mechanisms pave the way for innovative models for psychiatric disorders. In addition, fish are emerging models of social behaviour and associated pathological alterations. Sociality is a fundamental

dimension of human interactions and altered social behaviour is a symptom of many psychiatric disorders. In fish, due to the widespread influence of ecological and ecophysiological research, the focus has been largely aggressive behaviour – a symptom of acute psychotic states, drug abuse and mood disorders. A recent review highlighted that the broader array of social behaviour and cognition – from social motivation, social anxiety and social avoidance through social cognition, reproductive behaviour, and altruism and cooperation – represent important endpoints which could be used as models of specific disordered domains of psychiatric conditions related to mood disorders and social anxiety (Soares et al., 2018). Understanding how the microbiome interacts with these factors could open avenues for investigation on probiotics and other live biotherapeutics on psychiatric disorders (Marchesi et al., 2015; O'Toole, Marchesi, & Hill, 2017).

#### Box 2. The social life of farmed fish: the search for new boundaries of social learning and stress response

Fish farming is crucial for maintaining global protein sources for rising human consumption and for providing animals that can restock exploited or/and degraded habitats or for create new fishing areas (Fernö, Huse, Jakobsen, & Kristiansen, 2006). When these hatchery-reared fish, however, are released into the wild they tend to have low survival partially due to behavioural deficits, although other causes (and consequences) are still under debate (Young, 2013; NASCO, 2017). Overall, available information indicates that this is a product of rearing environment, which limits fish exposure to natural stimuli and has profound consequences in fish learning and decision-making skills. In summarizing the differences (and implications) between natural and farmed fish, we identify the following:

1. Behavioural shifts. i) Foraging: fish reared in restricted conditions are less able to adjust to novel situations, which may occur when reared fish are released into the wild, needing to identify and catch live prey; ii) Aggression and reproduction: implications of rapid growth and high density.

1. Spatial dynamics. Farmed fish live in high density groups, but because these constantly changing, the establishment of clear individual and collective behaviour may thus be limited. While exposure to a greater number of conspecifics could be a source of extra information and learning, in practice the ability to recognize and associate with conspecifics may also decrease with group size for some species (Griffiths & Magurran, 1997; Ward, Holbrook, Krause, & Hart, 2005). Additionally, artificial grouping of similar sized animals may also modulate behaviour.

1. Coping and anti-predator responses. The monocultural and relatively sterile conditions of aquaculture tend to produce fish unable to appropriately cope with novel and complex (natural) environments. The lack of previous exposure to predators, limits individual recognition which may become fatal (Brown & Warburton, 1999).

2. Parasite resistance. Stressful conditions can lead to immunocompromised fish that are more prone to infection and directly transmitted pathogens can spread rapidly amongst high density hosts.

3. Poorer development and physiological state (ontogeny), due to differences in nutrition, exercise and putative chronic stress levels of tank reared fish. Indeed, a combination of factors during the development of these fish, such as high levels of stress exposure and absence of appropriate socio-environmental stimulation may lead to limited neural and sensory development (Fernö et al., 2006).

In addition, many farmed fish are genetically selected, typically targeting rapid growth traits, which may also result in behavioural changes. In this respect, condition of the skin microbiota could also play a role in their behavioural neurobiology and welfare. For instance, farmed fish are more susceptible to pathogens compared to wild fish (e.g. Pulkinen et al), which may be mediated via changes to their microbiomes (but

see Minniti et al., 2017). Thus, the implications of fish domestication in terms of behavioural, microbial and physiological variation urgently warrants further research.

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